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Divergent pathways for TNF and C_2-ceramide toxicity in HTC hepatoma cells

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ABSTRACT

We previously showed that, in the rat hepatoma cell line HTC, TNF brings about a non-caspase-dependent, apoptosis-like process requiring NADPH oxidase activity, an iron-mediated pro-oxidant status, and a functional acidic vacuolar compartment. This process may thus involve mechanisms such as autophagy or relocation of lysosomal enzymes, perhaps secondary to the formation of ceramide by acidic sphingomyelinase. Here we investigated whether ceramide formation contributes to the apoptogenic process. HTC cells were found to be sensitive to exogenous ceramide and significantly protected against TNF by desipramine, an inhibitor of lysosomal acid sphingomyelinase. However, Bcl-2 transfection and Bcl-x_L upregulation by dexamethasone significantly diminished the apoptogenic effect of ceramide but not that of TNF, suggesting that ceramide is not directly involved in TNF toxicity. Moreover, Bcl-x_L silencing precluded dexamethasone-induced protection against ceramide and, by itself, induced massive death, demonstrating the strict dependence of HTC cells on Bcl-x_L for survival also under standard culture conditions.

1. Introduction

Primary hepatocellular carcinoma (HCC) is a malignancy with bad prognosis because of lack of effective treatments [1]. Although its etiology has been much looked for, and several risk factors identified, the molecular mechanisms that drive the neoplastic transformation of hepatocytes are still poorly understood.

Malignant transformation is a multiphase process by which cells accumulate mutations in relevant oncogenes and oncosuppressor genes that confer a selective growth advantage and, consequently, promote uncontrolled expansion of privileged clones. Whereas excessive proliferation relies on the upregulation of positive signals or the down-regulation of negative ones, insufficient cell deletion (*e.g.*, programmed cell death) that would counteract proliferation may reflect defects in the extrinsic (receptor-mediated) or intrinsic (mitochondria-triggered) apoptotic pathways. Hepatocytes have been categorized as type II cells [2], i.e. cells that require a tight cooperation and integration of both these apoptotic pathways to undergo programmed cell death following ligation of membrane death receptors. Consequently, aberration in either one of these pathways can affect the capability of HCC cells to undergo apoptosis following appropriate stimuli, resulting in resistance to programmed cell death. Since most chemotherapeutic agents rely on the induction of apoptosis for their action, such resistance may account for the unfavorable prognosis of HCC patients.

Among the many apoptogenic stimuli in mammalian cells, TNF has long been regarded as one of the most effective and, thus, it has been studied intensively. This particular cytokine can trigger different signaling cascades, which often converge by activating the caspase cascade and thereby apoptosis, although other forms of cell death have also been reported [3]. However, a number of both normal and neoplastic cells display strong resistance to TNF as well as other cytotoxic agents. Usually, this is due to various mechanisms of apoptosis suppression.

HTC cells, an established line of rat hepatoma cells, are resistant to several apoptogenic agents, TNF included, and may dominantly confer death resistance to somatic hybrids generated by fusion with otherwise sensitive cells [4].

We have previously reported that HTC cells can be sensitized to TNF by the simultaneous exposure to low concentrations of cycloheximide. This combined treatment causes a non-necrotic, apoptotic-like form of cell death that, interestingly, requires a properly functioning lysosomal compartment as well as normal NADPH oxidase activity [5]. Apart from occasional reports, which implicate stimulation of autophagy [6], the role of lysosomes in TNF cytotoxicity is

Abbreviations: 3-MA, 3-methyladenine; AO, acridine orange; BafA1, bafilomycin A1; C_2-cer, C_2-ceramide; CHX, cycloheximide; Dex, dexamethasone; Dpm, desipramine; Fumo, fumonisin B1; PS, phosphatidylserine; PI, propidium iodide; aSMNase, acidic sphingomyelinase; TNF, tumor necrosis factor

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supposed to involve the relocation of acidic proteases to the cytosol secondary to loss of lysosomal membrane integrity. One way this may occur is generation of the apoptogenic substance ceramide in the acidic endo-lysosomal compartment [7]. Ceramide, in turn, may also be cleaved by an acidic lysosomal ceramidase under formation of the lysosomotropic detergent sphingosine, which labilizes lysosomes if present in high enough concentrations [8].

This possibility, which is the object of the present investigations, is supported by our preliminary observation that desipramine (Dpm), an inhibitor of the acidic sphingomyelinase (aSMNase), protects HTC cells from the cytotoxic effect of TNF. To clarify the role of ceramide, we here compared the mechanisms behind the cytotoxic actions of TNF and ceramide on HTC cells.

2. Materials and methods

2.1. Cell cultures and treatments

HTC cells were grown in a 1:1 mixture of DMEM/Ham's F12 with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For experiments, cells were plated at $10^4/cm^2$ and, 24 h later, exposed to 20 ng/ml human recombinant TNF (R&D Systems, Minneapolis, MN, USA) and 10 μg/ml cycloheximide (CHX) or to different concentrations of C_2-ceramide (Sigma-Aldrich, Milan, Italy).

Culture media and all other reagents and chemicals were from Sigma-Aldrich (Milan, Italy), except for z-VAD.fmk that was from Alexis-Biochemicals (San Diego, CA, USA).

2.2. Plasmids and transfections

The control or encoding human Bcl-2 pBABE vectors [9] were transfected in HTC cells for 16 h with the DOTAP reagent (Roche Applied Science, Monza, Italy). The cells were then returned to standard culture conditions and grown for another 24 h before selection with 10 μg/ml puromycin. While untransfected cells died in 4–8 days, the mock and Bcl-2-transfected grow well in the presence of puromycin, which was removed 24 h before further experiments. The expression of hBcl-2 was detected by western blotting, with an anti-Bcl-2 antibody (Santa Cruz Biotechnology, Heidelberg, Germany).

2.3. Detection and quantification of early apoptosis-like alterations

The apoptotic population was estimated by counting the fraction of cells that were positive for phosphatidylserine (PS) and negative for propidium iodide (PI). The assay was done as previously described [5] using the Annexin V/PI reagent (Bender MedSystem, Wien, Austria) coupled to flow cytometric analysis on a FACScan machine (Becton-Dickinson, Milan, Italy) as described before [5]. For each sample, at least 5000 cells were acquired and analyzed with the CellQuest software (Becton-Dickinson).

2.4. Analysis of mitochondrial membrane potential

Changes in the mitochondrial membrane potential were analyzed using the JC-1 (Invitrogen, San Giuliano Milanese, Italy) technique. The cells, washed and resuspended in PBS, were stained at room temperature with 5 μM JC-1 for 10 min in the dark. Red and green fluorescence were then measured with a FACScan flow cytometer and analyzed with the CellQuest software. For each sample, at least 5000 cells were analyzed.

2.5. Analysis of lysosomal acidity and morphology

The effects of desipramine (Dpm) and NH_4Cl on the acidic compartment were examined by the acridine orange (AO) technique [10]. The cells were incubated with 5 μg/ml AO in complete medium at 37°C for 15 min, rinsed with PBS, detached and analyzed by flow cytometry. For each sample, at least 10 000 cells were analyzed.

For microscopy, cells were grown on chamber slides and treated with 50 μM Dpm or 20 mM NH_4Cl for 6 h. Then, 5 μg/ml AO was added to the medium for 5 min at room temperature. Following two washes with PBS, cells were observed under Nomarski and fluorescence optics. Representative fields were recorded and subsequently processed with Photoshop, version 7.0.

In order to further analyze the effect of Dpm, macro-autophagy was prevented by adding 10 mM 3-MA to the cultures 1 h before Dpm (50 μM). Moreover, to prevent the supposedly lysosomotropic drug Dpm to concentrate in the lysosomal compartment, cells were exposed to the proton pump inhibitor BafA1 (1 μM) before Dpm addition.

2.6. Bcl-x_L expression

For Bcl-x_L detection, cells were sonicated in lysis buffer (20 mM TRIS–Cl, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100). 20 μg of the lysates were resolved on a 12% PAGE. After blotting, the membranes were incubated overnight at 4°C with a rabbit polyclonal anti-Bcl-x_L (1:500, sc-1041, Santa Cruz Biotechnology) or for 1 h at room temperature with a mouse monoclonal anti-β-actin (1:2000, clone AC-15, Sigma-Aldrich) antibody. The blots were then washed with TBS-Tween 0.05% and incubated with the appropriate anti-IgG antibody (1:10 000, Bio-Rad Laboratories, Milan, Italy). Chemiluminescent detection was performed with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

For equal loading of lanes, the protein content of each sample was determined with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories).

Quantification of Bcl-x_L expression was obtained with the TotalLab TL100 analytical software (Nonlinear Dynamics, ver. 2006c, Newcastle upon Tyne, UK) and expressed as normalized intensity of the band of interest with respect to the amount of β-actin for each sample.

2.7. Downregulation of Bcl-x_L by RNA interference

Bcl-x_L was silenced using conditions that efficiently knock down Bcl-x_L in HTC cells [11]. 100 nM of the Bcl-x_L specific and control siRNAs (MWG-Biotech AG, Ebersberg, Germany) were transfected with Metafectene Pro (Biontex, Martinsried-Planegg, Germany) for

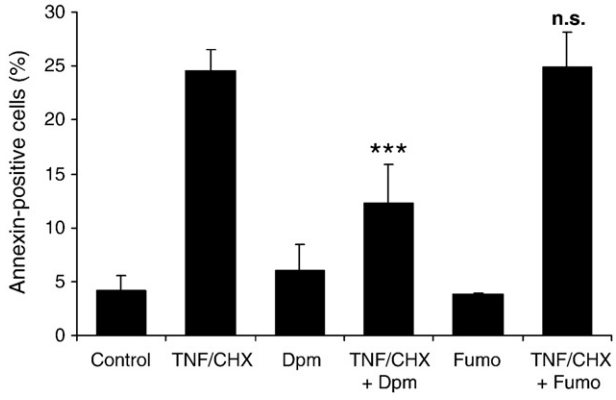

Fig. 1. Effect of desipramine and fumonisin on cell death. HTC cells were exposed for 6 h to 20 ng/ml TNF and 10 μg/ml CHX, in the presence of 50 μM desipramine or fumonisin B1 and subsequently analyzed by flow cytometry. Early apoptosis-like cell alteration was quantified as the proportion of cells exposing phosphatidylserine and excluding propidium iodide. For each sample, at least 5000 cells were analyzed. Results represent the means ± SD of three independent experiments. Statistical significance of the differences was evaluated by one-way ANOVA test, followed by the Student–Newman–Keuls post test. ***: $p<0.001$ *vs* TNF/CHX. n.s.: not significantly different *vs* TNF/CHX.

4 h in Opti-MEM I (Invitrogen). The cells were returned to standard conditions for another 20 h and then treated with C_2-cer or TNF/CHX for 24 or 6 h in the presence or absence of dexamethasone (Dex) before being harvested. Cell death and Bcl-x_L levels were quantified as described above.

2.8. Statistical analysis

The shown results represent the means ± SD from at least three independent experiments. Statistical significance of the differences between groups was analyzed by one-way ANOVA and the Student–Newman–Keuls post test using the Instat software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Mechanisms of ceramide-induced cell death in HTC cells

We have previously shown that TNF/CHX treatment is cytotoxic for HTC cells. TNF toxicity was significantly diminished by bafilomycin A1 and ammonium chloride, which both raise the endo-lysosomal pH. Therefore, a properly functioning acidic compartment appeared to be an essential requirement for TNF/CHX-induced cell death. The contribution of this compartment to a death receptor-mediated cytotoxicity may rely on various molecular mechanisms [12,13], among which ceramide generation has been suggested as an important, though not exclusive occasion [14].

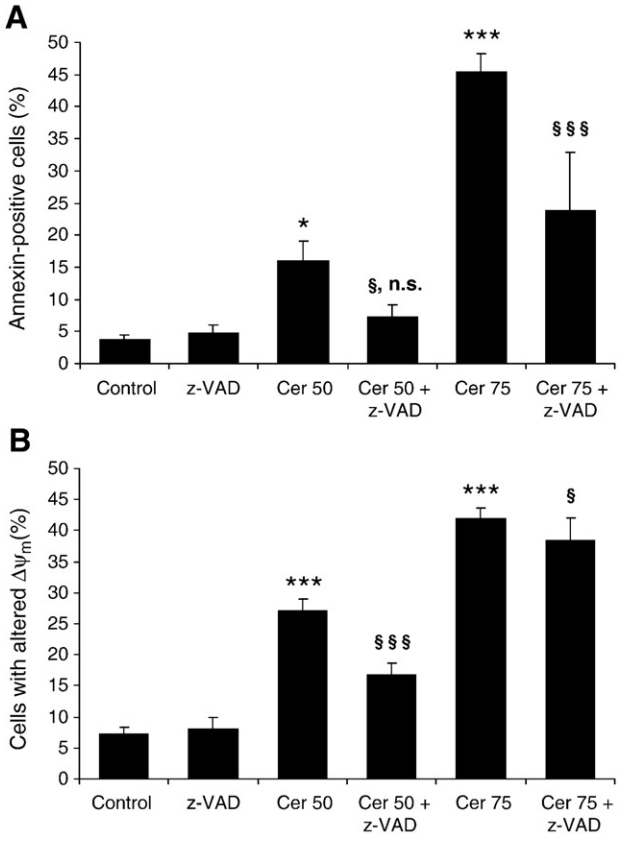

Fig. 2. Characterization of ceramide-induced cell death. (A) HTC cells were exposed for 24 h to 50 and 75 μM C_2-cer and analyzed by flow cytometry using the annexin V/propidium iodide (PI) test as in Fig. 1. The percentage of necrotic cells was in the range of 5–7% with no appreciable changes due to treatments. (B) The alteration of mitochondrial membrane potential following C_2-cer treatment (as in A) was evaluated by the JC-1 technique. In both experiments, the pan-caspase inhibitor z-VAD.fmk (50 μM) was added 1 h before C_2-cer. For each experiment, at least 5000 cells were acquired and analyzed. Data and statistical analysis as in Fig. 1. * and ***: $p<0.05$ and $p<0.001$ *vs* controls. § and §§§: $p<0.05$ and $p<0.001$ *vs* C_2-cer at the indicated concentrations. n.s.: not significantly different *vs* controls.

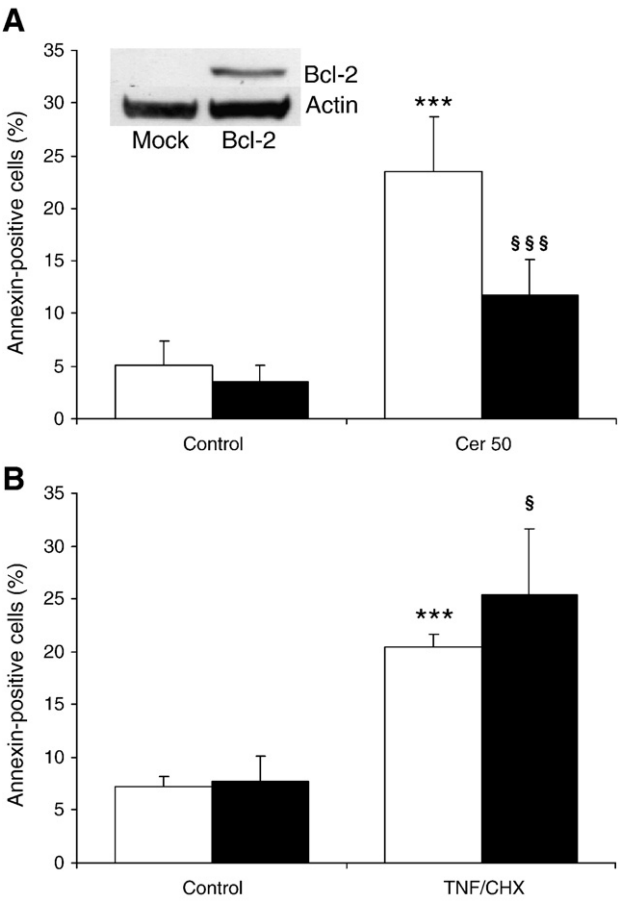

Fig. 3. Effect of Bcl-2 on ceramide and TNF toxicity. HTC cells were stably transfected with a retroviral vector encoding a human Bcl-2 cDNA (dark bars) and treated with either 50 μM C_2-cer for 24 h (A) or TNF/CHX as before for 6 h (B). As a control, the empty vector was stably transfected (empty bars). After treatment, cells were stained with annexin V/PI and analyzed by flow cytometry. For each experiment, at least 5000 cells were acquired and analyzed. Data and statistical analysis as in Fig. 1. The insert in A shows the expression of human Bcl-2 in control (Mock) and Bcl-2 transfected cells (Bcl-2). ***: $p<0.001$ *vs* controls. §§§ and §: $p<0.001$ and $p<0.05$ *vs* mock-transfected cells.

To explore whether ceramide contributed to TNF/CHX-dependent death, HTC cells were treated with this combination of drugs in the presence of desipramine (Dpm, 50 μM) or fumonisin B1 (Fumo, 50 μM), which respectively inhibit aSMNase and ceramide synthase. Whereas no effect of Fumo was detected, Dpm significantly prevented cell death (Fig. 1). This finding may be compatible with the hypothesis that the formation of ceramide by aSMNase contributes to cellular death at least to some extent, although in other experiments (not shown) no protection was afforded by Dpm at lower concentrations (5 and 20 μM), usually adequate to protect cells from ceramide generated in the acidic compartment.

To verify this hypothesis, the sensitivity of HTC cells to exogenous ceramide was evaluated. The cells were treated with different concentrations of C_2-ceramide (C_2-cer) for 24 h when it was found that C_2-cer had produced a well defined phosphatidylserine-positive population (Fig. 2A). As inferred from the protection afforded by the pan-caspase inhibitor z-VAD.fmk at 50 μM (Fig. 2A and B) or 20 μM concentration (not shown), caspases were involved in this C_2-cer-induced death (type I programmed cell death). By contrast, we found caspase inhibitors totally ineffective

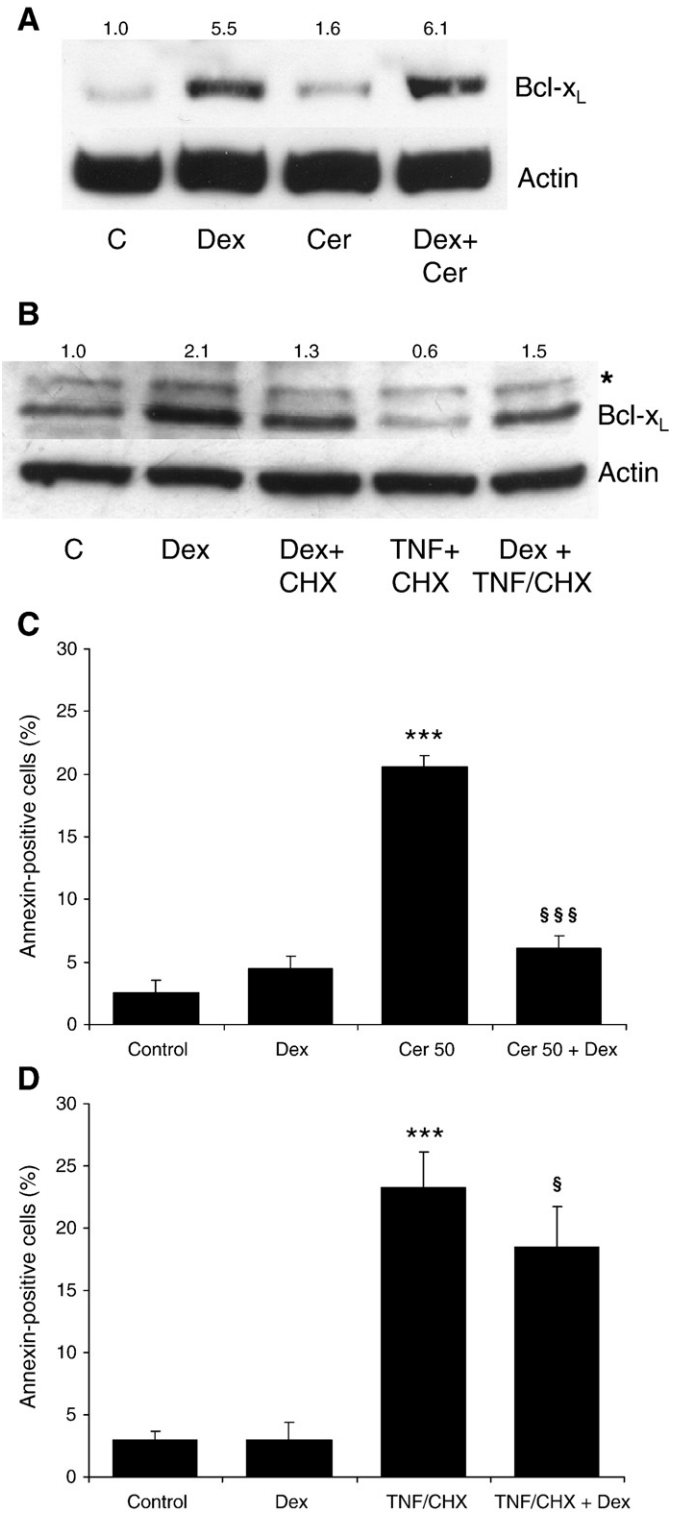

Fig. 4. Effect and molecular mechanism of protection by dexamethasone from ceramide and TNF toxicity. HTC cells were incubated for 18 h with 1 μM Dex, and subsequently with 50 μM C2-cer (A, C) or TNF/CHX as before (B, D) for 24 and 6 h, respectively. Bcl-x_L (29 kDa) was measured by western blotting (A, B), and cell death by flow cytometry by exposure to annexin V/PI (C, D). At least 5000 cells were analyzed for each condition. Changes in the relative abundance of Bcl-x_L with respect to control cells are indicated. Actin: β-actin band (42 kDa); the asterisk in panel B indicates non-specific bands due to antibody cross-reactivity. Data and statistical analysis as in Fig. 1. ***: $p<0.001$ *vs* controls. §§§ and §: $p<0.001$ and $p<0.05$ *vs* 50 μM C_2-cer (panel C) or TNF/CHX (panel D).

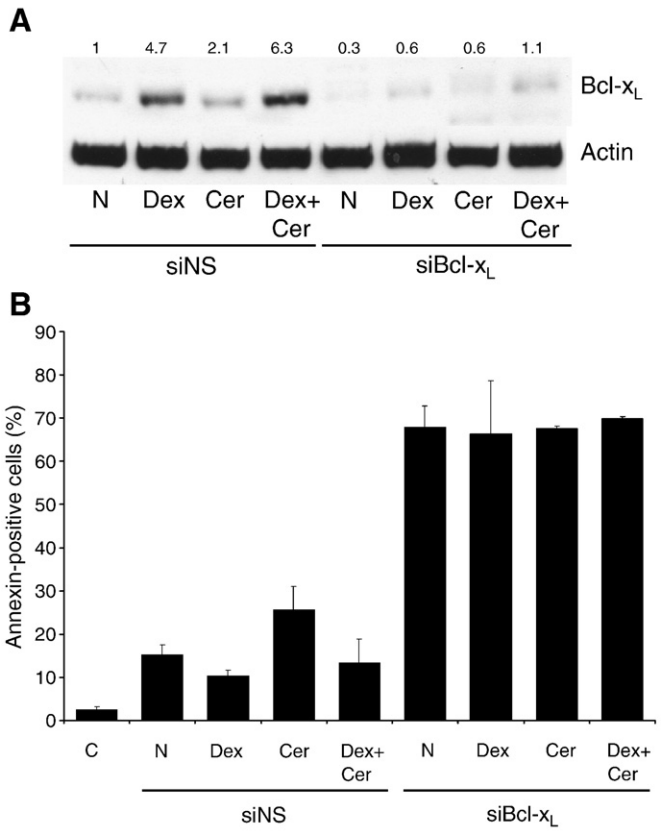

Fig. 5. Downregulation of Bcl-x_L and its effects on cell death. To eradicate Bcl-x_L, HTC cells were transiently transfected with sequence-specific siRNAs duplexes. As a control, a non-specific duplex with a GC content similar to that of specific siRNAs was used. Silenced and control cells were treated for 24 h with 50 μM C_2-cer and 1 μM Dex as indicated, and subsequently harvested for Bcl-x_L detection by western blotting (A) or for FACS analysis after staining with annexin V/PI (B). The data represent the means ± SD of two independent experiments, assayed in triplicate. siNS: non-specific silencing duplexes; siBcl-x_L: siRNAs specifically targeting Bcl-x_L sequence. Changes in the relative abundance of Bcl-x_L with respect to non-silenced cells are indicated.

against TNF toxicity on the same cells [5]. Moreover, the increased proportion of cells with altered mitochondrial potential (Fig. 2B) suggests that caspase activation was triggered by the intrinsic apoptotic pathway.

3.2. Effects of Bcl-2 expression and dexamethasone on C_2-cer- and TNF-induced cell death

Bcl-2 and other anti-apoptotic proteins of this family, including Bcl-x_L, protect against apoptosis triggered by several apoptogenic agents. In particular, Bcl-2 depresses the effects of ceramide in various experimental models [15]. Thus, we investigated the effects of ectopically expressed Bcl-2 (Fig. 3A, insert) on the toxicity elicited by TNF/CHX and C_2-cer, the latter used at the lowest effective concentration (50 μM). Bcl-2- and mock-transfected HTC cells were treated with TNF/CHX or C_2-cer for 6 and 24 h, respectively. Stable Bcl-2-transfectants were significantly protected from C_2-cer (Fig. 3A), but not from TNF/CHX (Fig. 3B). Therefore, ceramide toxicity progressed through a Bcl-2-sensitive pathway, while TNF/CHX toxicity took place through a Bcl-2-insensitive one.

To validate this hypothesis, HTC cells were exposed to TNF/CHX or C_2-cer in the presence of dexamethasone (Dex), a synthetic glucocorticoid that, via Bcl-x_L upregulation, protects HTC cells from apoptosis by serum starvation or UV-C [11]. HTC cells exposed to Dex for 18 h underwent a clearcut elevation of the Bcl-x_L protein,

irrespective of subsequent treatments (Fig. 4A–B). CHX, which is required to sensitize the cells to TNF, only slightly reduced this increase (Fig. 4B). However, only the C_2-cer-induced cell death was markedly suppressed by Dex, while the protection against TNF/CHX was only marginal (Fig. 4C–D), further confirming that TNF- and C_2-cer-induced toxicity rely on different mechanisms. In addition, this finding also proves that Bcl-x_L upregulation, similarly to ectopic Bcl-2 expression, can afford protection from selected death stimuli in HTC cells.

Dpm, like other tricyclic antidepressants, has been reported to raise the intracellular concentration of glucocorticoids by inhibiting their extrusion [16], and could thus mimic the effect of Dex on the Bcl-x_L levels. However, in HTC cells the intracellular Bcl-x_L levels were not modified by Dpm (data not shown). To further demonstrate that Bcl-x_L was responsible for the protection afforded by Dex against C_2-cer, we reduced its level by the RNA interference technique. Bcl-x_L-specific siRNAs caused the Bcl-x_L protein to decrease to almost undetectable levels in HTC cells, whether exposed or not to Dex (Fig. 5A). Under these conditions, however, Dex did not further prevent C_2-cer-induced cell death, thus confirming the criticality of Bcl-x_L in suppression of ceramide-induced death (Fig. 5B). Noteworthily, siRNA-mediated depletion of Bcl-x_L by itself elicited cell death, independently of any additional cytotoxic treatment.

Altogether, the above results support the view that TNF toxicity on HTC cells is not mediated by ceramide. Consequently, they also imply that the protection afforded by Dpm against TNF/CHX cannot be ascribed to interference with ceramide metabolism.

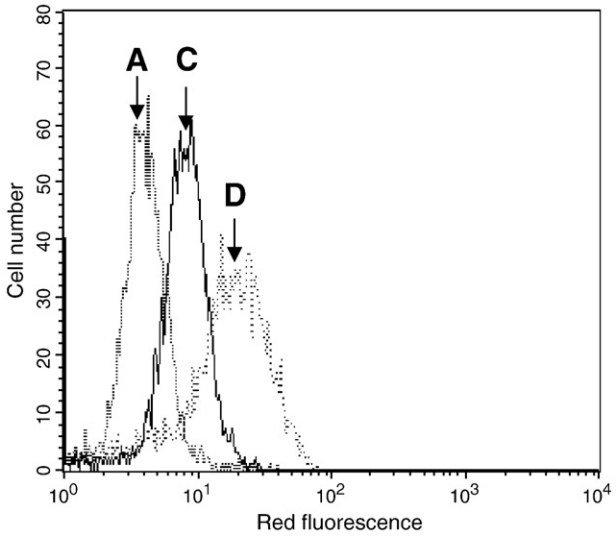

Fig. 7. Effect of NH_4Cl and desipramine on the lysosomal compartment. HTC cells were treated for 6 h with 20 mM NH_4Cl (A) or 50 μM desipramine (D). Control cells (C) did not receive any treatment. After 15 min loading with 5 μg/ml acridine orange at 37 °C, cells were detached by trypsin and immediately analyzed by flow cytometry. Histograms represent red fluorescence collected from at least 10000 cells for each condition. Note declined AO uptake following exposure to NH_4Cl (A) as a result of lysosomal alkalinization, while the broad, right-deviated peak following exposure to Dpm (D) suggests a largely expanded lysosomal compartment. Data acquisition and analysis were performed with CellQuest Analytical Software.

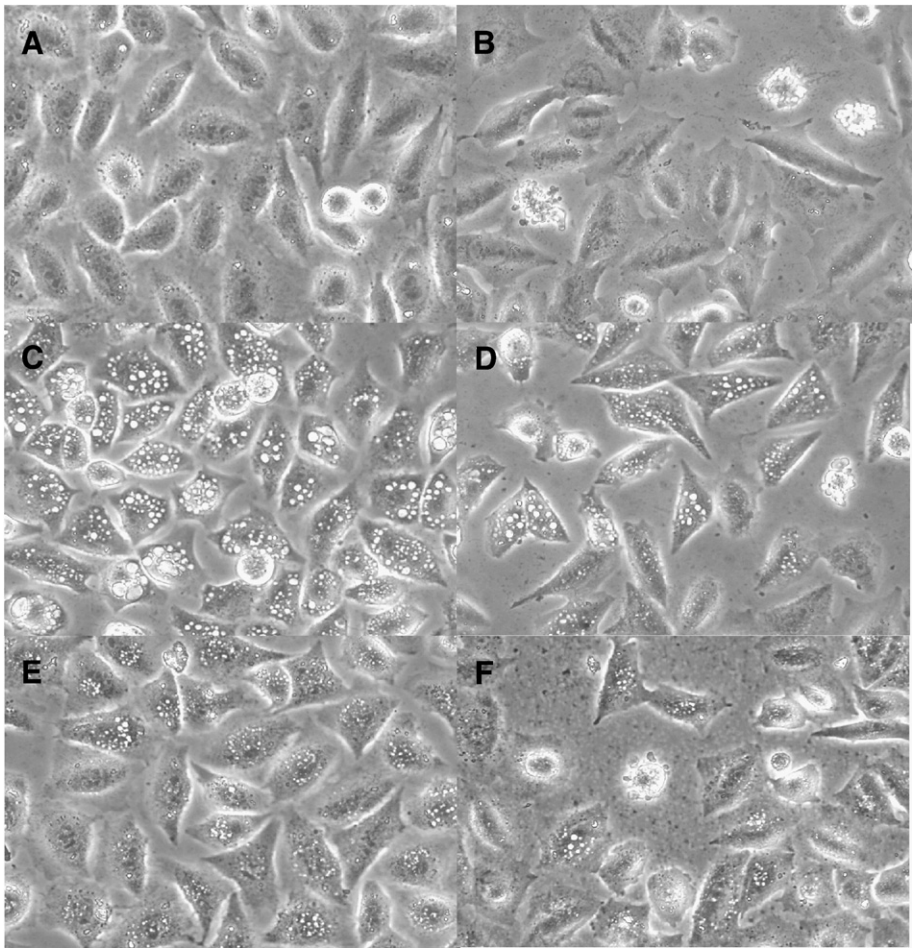

Fig. 6. Effect of desipramine and ammonium chloride on cell morphology. HTC cells were treated for 6 h with TNF/CHX alone (B) or in the presence of either 50 μM desipramine (D) or 20 mM NH_4Cl (F). As controls, cells were left untreated (A) or exposed to Dpm (C) or NH_4Cl (E) alone. Note heavy and moderate vacuolization of cells after exposure to Dpm and NH_4Cl, respectively. Phase-contrast pictures (magnification 40×) were recorded with a digital camera and details representative of each condition composed with Photoshop, version 7.0.

3.3. Effect of desipramine on the endo-lysosomal compartment

While exploring the possible mechanisms by which HTC cells are protected by Dpm from TNF/CHX-induced death, we noticed that the drug affected cell morphology and caused the appearance of a number of variously-sized, optically clear vacuoles (Fig. 6C–D). Such vacuoles were similar to, albeit more prominent than, those observed in cells treated with NH_4Cl (Fig. 6E–F), an agent that alkalizes lysosomal pH. We compared cells treated for 6 h with Dpm or NH_4Cl for changes in their lysosomal compartment, using flow cytometry and light microscopy following staining with the lysosomotropic fluorochrome acridine orange (AO). As expected, the red fluorescence was significantly reduced by NH_4Cl (Fig. 7). By contrast, Dpm shifted the fluorescence to values definitely higher than in controls. By Nomarski and fluorescence microscopy (Fig. 8), control cells (panels A–C) displayed a number of small red fluorescent granules corresponding to lysosomes. In NH_4Cl-treated cells (panels D–F), which were slightly rounded up, many of the fluorescent granules were moderately enlarged, while their total number appeared somewhat decreased (perhaps because of their lower detectability due to the pH increase). Dpm-treated cells were characterized by large and intensely red lysosomes (in good agreement with the FACS data) and, on image overlays, they perfectly matched the optically clear vacuoles (panel G–I, arrowheads). Obviously, Dpm causes an expansion of the lysosomal compartment and a change from many small lysosomes to a few large ones. We considered the possibility that these alterations reflect enhanced autophagy or osmotic swelling of the lysosomes. The depression of macro-autophagy by 3-MA for a short period of time neither affected the cell morphology nor altered the vacuolization caused by Dpm (Fig. 9). By contrast, this vacuolization was completely suppressed on alkalinization of the lysosomal compartment by BafA1. This result strongly indicates that Dpm is a highly lysosomotropic drug that accumulates in the endo-lysosomal compartment raising its osmotic pressure and, thereby, inducing the formation of large vacuoles under consideration.

4. Discussion

TNF, one of the most powerful inducers of apoptosis and necrosis, may trigger different mechanisms that lead to caspase activation, which in turn results in classic apoptotic cell death [2]. This particular cytokine, however, can also elicit additional kinds of non-necrotic cell death that substantially differ from conventional apoptosis.

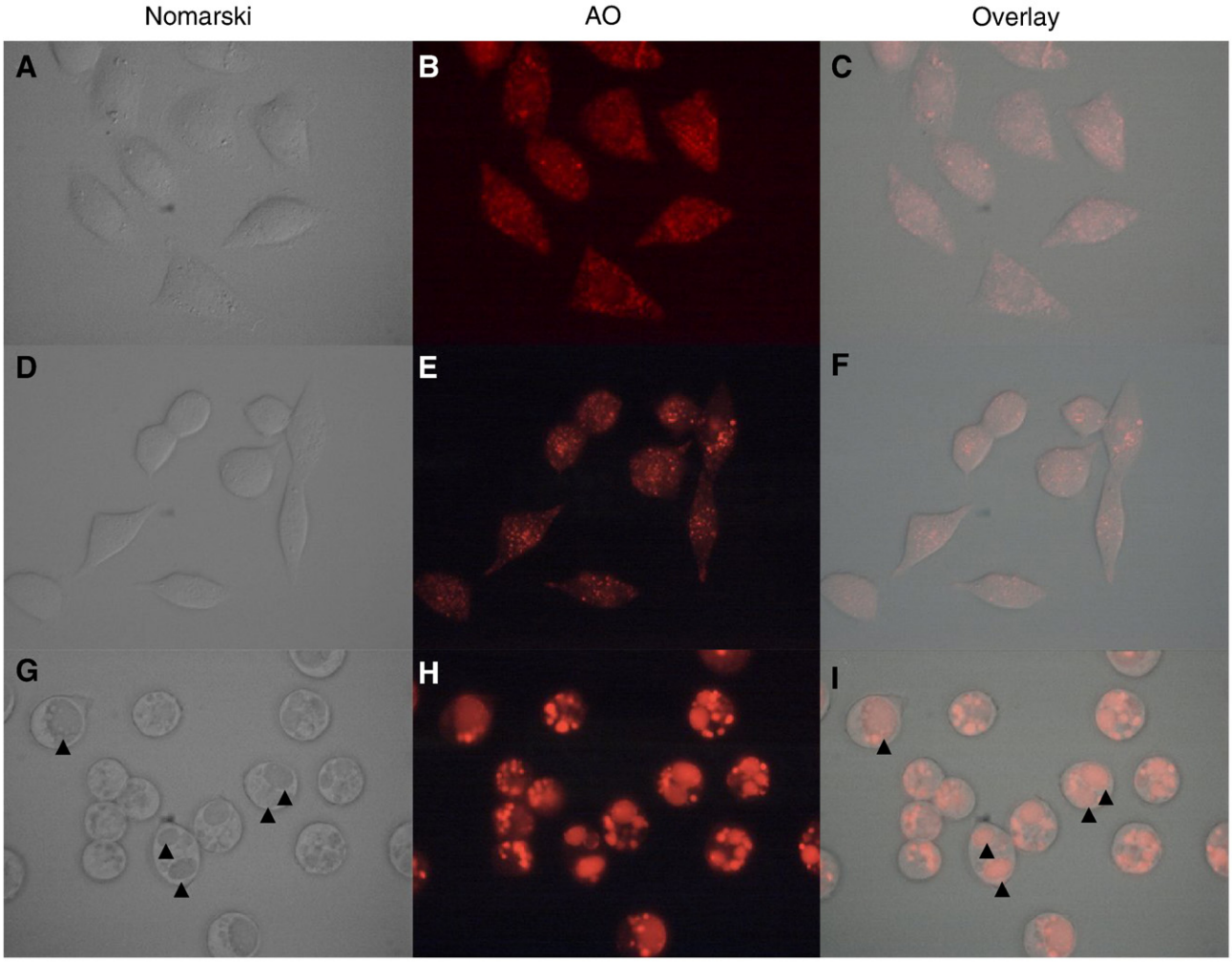

Fig. 8. Structural and morphological alterations of the lysosomal compartment. Cells were exposed to 20 mM NH_4Cl or 50 μM desipramine for 6 h. Following a 5 min long ensuing exposure to 5 μg/ml acridine orange at room temperature, samples were observed by Nomarski (panels A, D, G) and fluorescence microscopy (panels B, E, H). A–C: control cells; D–F cells treated with NH_4Cl; G–I cells treated with Dpm. Representative fields were acquired with a digital camera and overlaid (C, F, I) using Photoshop. Note slightly rounded up cells following exposure to NH_4Cl. Cells exposed to Dpm are rounded up, partly degenerated and show numerous enlarged lysosomes, suggestive of osmotic swelling. Arrowheads (panels G and I) demonstrate that the large and optically clear vacuoles resulting from desipramine treatment match with the intense red fluorescent acidic vesicles.

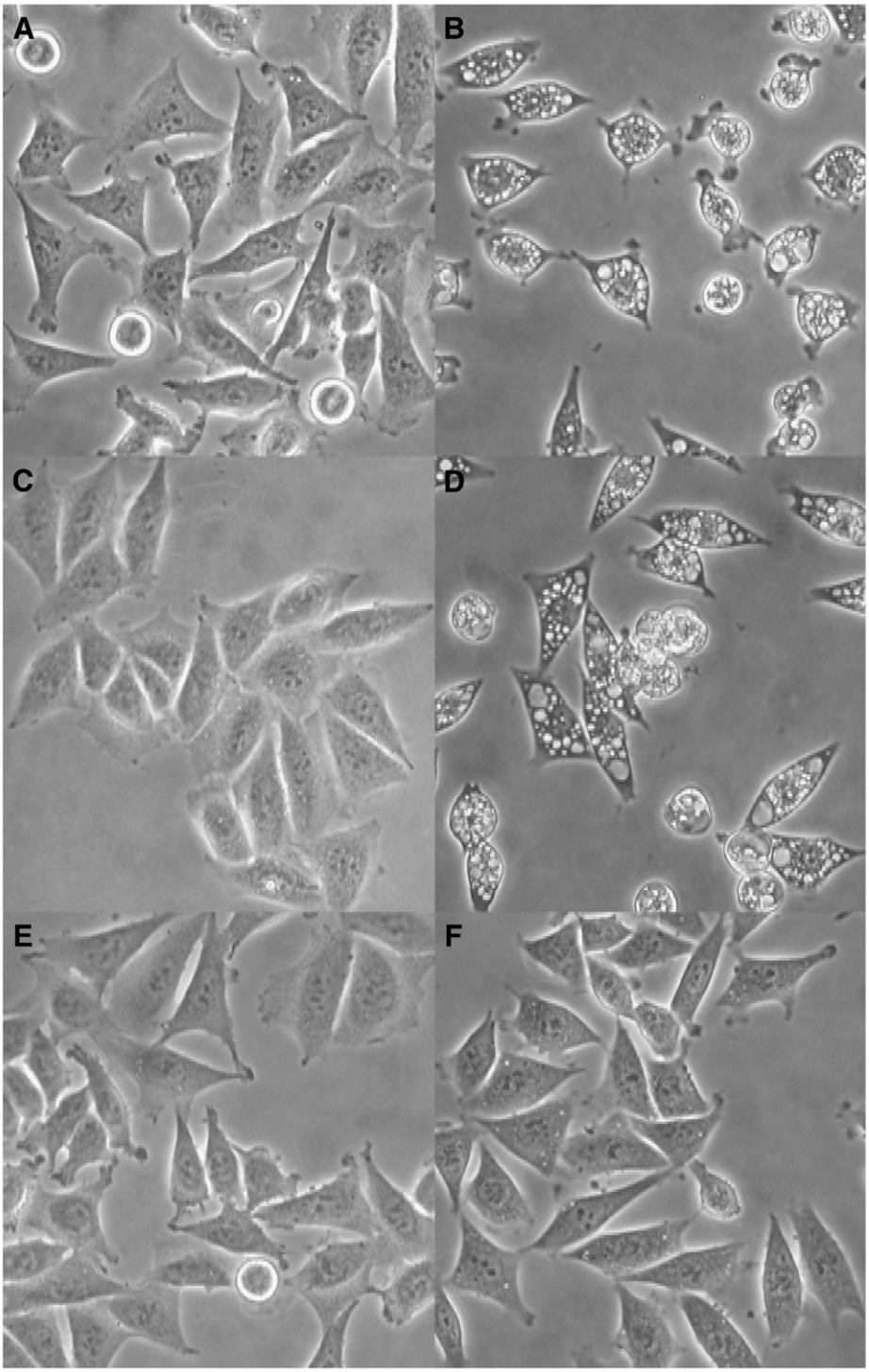

Fig. 9. Effect of 3-MA and BafA1 on desipramine-induced vacuolization. HTC cells were treated for 4 h with 50 μM desipramine in the presence of 10 mM 3-MA (D) or 1 μM BafA1 (F). As controls, cells were left untreated (A) or exposed to Dpm (B) or 3-MA (C) and BafA1 (E) alone. Both 3-MA and BafA1 were added to the cultures 1 h before the addition of Dpm.

In this context, we have previously reported that in HTC cells TNF/CHX brings about an atypical apoptosis-like but caspase-independent death mode [5], in which the acidic vacuolar compartment as well as NADPH oxidase is involved. NADPH oxidase was found implicated also in TNF-induced necrotic death of L929 cells [17]. Apart from the well-known role in the autophagy-associated cell death (reviewed in [18]), the involvement of lysosomes in death pathways mostly proceeds through membrane labilization, with consequent release of hydrolases to the cytoplasm [19] (reviewed in [20]) or via ceramide generation due to the action of aSMNase [7,21]. TNF may cause death by either of these mechanisms [22]. In our previous report [5], using standard techniques we could, however, detect neither any increase in total cathepsin activity nor release of lysosomal enzymes to the cytosol in TNF-treated HTC cells. In addition, selective cathepsin inhibitors did not suppress TNF toxicity, although drugs that raise lysosomal pH, such as bafilomycin A and ammonia did.

In the present study, we investigated whether any generation of ceramide by aSMNase is involved in the TNF-triggered death of HTC cells. The protection from TNF/CHX that was afforded by Dpm, which is known to suppress ceramide generation in the acidic compartment

[23], indeed suggested the involvement of this lipid mediator in death signal transduction. However, while HTC cell death induced by TNF/CHX previously was found to be both caspase- and mitochondria-independent, we here show that, in contrast, death by ceramide is both caspase- and mitochondria-dependent. It follows that cell injury by these two agents proceeds through unrelated pathways, thereby implying that Dpm protects from TNF by mechanisms other than interference with ceramide metabolism and aSMNase activity. Since we observed that Dpm is a lysosomotropic drug that causes alterations of the acidic vacuolar compartment of HTC cells due to osmotic swelling owing to its accumulation in the lysosomal compartment [24], it seems probable that its protective effect is mediated by an interference with the lysosomal compartment that prevent its normal functioning — something that seems to be a requirement for TNF/CHX.

In agreement with previous reports, HTC cells express no detectable levels of Bcl-2 [25], which is one of the most protective factors against agents that activate the intrinsic apoptotic pathway, ceramide included [15]. As expected, enforced Bcl-2 expression indeed protected HTC cells from ceramide. By contrast, ectopic Bcl-2 expression afforded no protection against TNF/CHX exposure, but rather somewhat increased the sensitivity to this cytokine. This finding clearly lends further support to the view that TNF and ceramide trigger diverse death mechanisms in HTC cells.

Consistent evidence was provided by experiments using Dex, a synthetic glucocorticoid, which is known to both induce [26] and protect from [27] apoptosis, depending on the cell type. The anti-apoptotic effect of Dex relies on upregulated expression of protective factors such as Bcl-2 and Bcl-x_L [28,29], and this mechanism has been shown to be effective in HTC cells as well [11]. In the present study, Dex did not attenuate TNF toxicity despite an efficient Bcl-x_L induction, but completely abrogated ceramide toxicity. This protection can be ascribed to Bcl-x_L, since protection was no longer observed following Bcl-x_L silencing, when the cells rather underwent massive death. Since upregulation of Bcl-x_L, or enforced expression of Bcl-2, both prevented ceramide-induced cell death, our results also suggest that these two Bcl-2 family members can substitute for each other in shutting down signals that trigger the intrinsic death pathway and in granting increased death resistance to hepatoma cells.

In conclusion, the results presented in this study strongly support the view that, even if dependent on the endo-lysosomal apparatus and effectively prevented by Dpm, TNF/CHX toxicity to HTC cells does not involve ceramide as a mediator. Further it is caspase- and mitochondria-independent and is antagonized by neither ectopic Bcl-2 expression, nor exposure to Dex, which upregulates Bcl-x_L expression. In contrast, cell death due to ceramide exposure proceeds via activation of caspases and of the intrinsic apoptotic pathway and is strongly antagonized by ectopic Bcl-2, or by the Bcl-x_L overexpression brought about by Dex.

These findings strongly argue against the notion that the attenuation of TNF toxicity afforded by 50 μM Dpm is accounted for by it being an aSMNase inhibitor. Rather the observation that this drug elicits pronounced morphological changes of the lysosomal compartment indicative of osmotic swelling suggests that the protective effect is due to interference with normal endo-lysosomal function.

Finally, though beyond the scopes of the present study, but in consistency with a previous report [11], we observed that Bcl-x_L silencing was sufficient to induce massive HTC cell death under completely normal culture conditions, indicating that these hepatoma cells are critically dependent on Bcl-x_L for survival. Interestingly, it was shown that Bcl-2 is undetectable in various human hepatoma cell lines, which, conversely, express high levels of Bcl-x_L [30]. Moreover, the same authors also reported that human HCCs have significantly higher Bcl-x_L and lower Bcl-2 levels than adjacent normal liver tissue, thus supporting the view that deregulated expression of Bcl-x_L contributes to the development of HCCs and suggesting that Bcl-x_L may be a significant therapeutic target.

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